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Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Heteropolyflavan-3-ols and Glucosylated Heteropolyflavans in Sorghum [*Sorghum bicolor* (L.) Moench]

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to characterize the structural diversity of polyflavans in Ruby Red sorghum [*Sorghum bicolor* (L.) Moench]. Deionization of the polyflavan fractions with Dowex 50 × 8−400 cation-exchange resin and subsequent addition of cesium trifluoroacetate (^{133}Cs) allowed the detection of exclusively $[M + Cs]^+$ ions. MALDI-TOF MS of the polyflavans that eluate from Sephadex LH-20 columns with methanol and acetone detected a series of masses corresponding to heteropolyflavan-3-ols differing in degree of hydroxylation and nature of the interflavan bond (A-type and B-type). MALDI-TOF MS of the Sephadex−ethanol/methanol (v/v) eluate revealed a series of masses corresponding to heteropolyflavan-5-*O*-β-glucosides that vary in the extent of hydroxylation and contain a flavanone (eriodictyol or eriodictyol-5-*O*-β-glucoside) as the terminal unit. The combination of liquid chromatographic separation and MALDI-TOF MS to characterize sorghum polyflavans indicates that the structural heterogeneity is much greater than previously described.

KEYWORDS: MALDI-TOF MS; sorghum; polyflavan; polyflavan-3-ol; proanthocyanidin

INTRODUCTION

Polyflavans are a class of flavonoids found in foods such as grapes, cranberries, chocolate, and sorghum. Proanthocyanidins and condensed tannins are synonyms of these oligomers. Polyflavans in sorghums have classically been regarded as antinutrients, being associated with reduced protein availability (*1*). However, more recent research suggests that polyflavans in foods might prevent diseases such as atherosclerosis, cancer (*2*), and urinary tract infections (*3*). Research on the role of polyflavans in health and nutrition is limited by the lack of analytical methods that relate the structural complexity of polyflavans found in foods and beverages to their biomedical effects. Because of the difficulty of isolating this class of compounds, polyflavans are often characterized as being homopolymers with uniform interflavan bonds and monomeric units (*4*).

Recent advances in mass spectrometry now allow for the characterization of complex mixtures of polyflavans. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is ideally suited for characterizing polydispersed oligomers (*5*). MALDI-TOF MS produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision (*6*). We have applied MALDI-TOF MS to characterize the structural diversity of polyflavans in foods and beverages (*7*, *8*). Grape seed extracts were shown to contain polyflavan-3-ols with gallic acid substitutions (*8*, *9*). Polyflavan-3-ols from *Lotus coniculatus* were found to contain heteropolymeric structures incorporating catechin/epicatechin and gallocatechin/epigallocatechin units (*10*). The presence of A-type interflavan bonds in cranberry (*7*, *3*) and brown soybeans (*11*) has been described. MALDI-TOF MS also provides a rapid method for characterizing the mass distribution of oligomeric polyflavans. Polyflavan-3-ols from apples have a mass distribution up to the undecamer (*12*), and the degree of polymerization (DP) of polyflavan-3-ols in brown or black soybean coat was found to be as high as DP30 (*11*).

Most of the research on sorghum [*Sorghum bicolor* (L.) Moench] described the polyflavans as homopolymers of catechin/epicatechin with uniform B-type interflavan bonds (*13*). However, Brandon et al. (*14*) found evidence of heteropolymers with both catechin/epicatechin and gallocatechin/epigallocatechin hydroxylation patterns (**Figure 1**). In addition, Gujer et al. (*15*) described the structure of unique sorghum polyflavan dimers and trimers, glucosylated on the 5-hydroxy group of the extending flavan units with a flavanone, either eriodictyol or eriodictyol 5-*O*-β-glucoside, as the terminal unit (**Figure 1**). Identification of these compounds shows that structural variations of polyflavans occur in the monomeric units (flavan extending units and a flavanone terminal unit) and the nature of substitutions (hydroxylation or glucosylation). We present results from MALDI-TOF MS studies that demonstrate that the structural diversity of sorghum polyflavans is much greater than previously appreciated.

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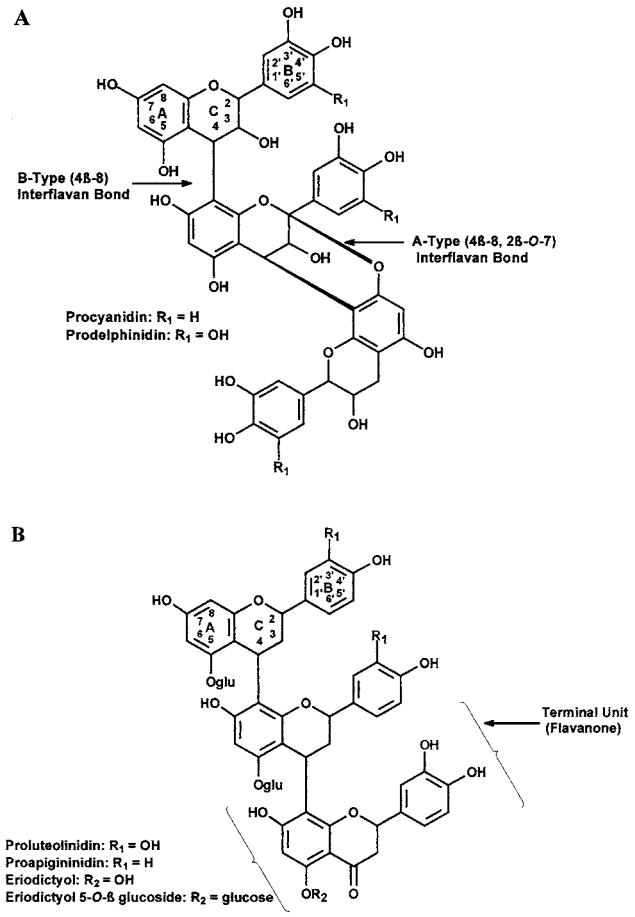

Figure 1. (A) Heteropolyflavan-3-ols from Ruby Red sorghum [*Sorghum bicolor* (L.) Moench]. (B) Glucosylated heteropolyflavans with a flavanone, eriodictyol or eriodictyol-5-*O*-β-glucoside, as the terminal unit from Ruby Red sorghum [*Sorghum bicolor* (L.) Moench].

EXPERIMENTAL PROCEDURES

Sorghum Extraction. Two grams of ground sorghum grain (Ruby Red; Natural Ovens, Manitowoc, WI) was extracted in 20 mL of 70% aqueous acetone (v/v) in an ultrasonic bath for 10 min. The extract was centrifuged (3500*g*) for 10 min and the liquid retained. Acetone was removed from the extract under vacuum evaporation at 30 °C. The extract was solubilized in 10 mL of ethanol.

Polyflavan Content of Grain. Ruby Red sorghum was characterized as a type III sorghum, because of the extractability of polyflavans in 70% aqueous acetone (v/v). Sorghum grain was analyzed for polyflavan content using the modified vannilin assay (*16*). The concentration of polyflavan (expressed as catechin equivalents) was 21.6 mg g^{-1}.

Sephadex LH-20 Separation. Sephadex LH-20 (Pharmacia) was equilibrated in ethanol for 2 h. A 10 cm × 2.5 cm i.d. Kontes glass preparative column was filled with Sephadex slurry to a height of 10 cm. The sorghum extract was applied to the column and eluted sequentially with ethanol (100 mL), ethanol/methanol (v/v; 100 mL), methanol (100 mL), and 80% aqueous acetone (v/v; 100 mL). The fractions were taken to dryness by vacuum evaporation at 30° C. Extracts were solubilized in 1 mL of 80% aqueous acetone prior to MALDI analysis.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Mass spectra were collected on a Bruker Reflex II-MALDI-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm). In the positive reflectron mode, an accelerating voltage of 25.0 kV and a reflectron voltage of 26.5 kV were used. In the positive linear mode, an accelerating voltage of 25.0 kV was used. Spectra are the sum of 300 shots. Spectra were calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW) as external standards.

In accordance with previously published results (*8*) *trans*-3-indoleacrylic acid (*t*-IAA; 5 mg/100 μL of 80% aqueous acetone) was used as a matrix. The polyflavan fractions eluted from the Sephadex column with methanol and 80% acetone were mixed with the matrix solution at volumetric ratios of 1:2. The polyflavan fraction eluted from the Sephadex column with ethanol/methanol was mixed with the matrix solution at a volumetric ratio of 1:1. The polyflavan/matrix mixture was either deionized and spiked with a solution containing a single cation (K^+, Na^+, Ag^+, or Cs^+) or applied directly (0.2 μL) to a stainless steel target and dried at room temperature. Dowex 50 × 8−400 cation-exchange resin (Supelco), equilibrated in 80% aqueous acetone (v/v), was used to deionize the analyte:matrix solution. NaCl or KCl (0.1 M, 0.5 μL), silver trifluoroacetate (0.01 M, 6 μL), or cesium trifluoroacetate (0.01 M, 6 μL) was added to the matrix/analyte solution to promote the formation of a single ion adduct ($[M + Na]^+$, $[M + K]^+$, $[M + Ag]^+$, $[M + Cs]^+$). Silver trifluoroacetate, KCl, NaCl, *t*-IAA (Aldrich Chemical Co., Milwaukee, WI), cesium trifluoroacetate, bradykinin, and glucagon (Sigma Chemical Co., St. Louis, MO) were used as received.

RESULTS AND DISCUSSION

Rationale for Assigning Structures to Mass. Tentative structures were assigned to sorghum polyflavans by comparing MALDI-TOF MS mass distributions to predictive equations. In the case of sorghum, Gupta and Haslam (*13*) described the polyflavan-3-ol oligomers as containing repeating units of catechin or epicatechin. Brandon et al. (*14*) later reported that a small proportion (8%) of the polyflavan-3-ol units exhibited a gallocatechin/epigallocatechin hydroxylation pattern, representing heteropolymers (**Figure 1**). The predictive equations describe the heteropolymeric nature of sorghum polyflavans on the basis of the assumption that the structural diversity seen in dimers and trimers (*13*−*15*) can be extrapolated to higher degrees of polymerization (DP) (**Tables 1** and **2**). Whereas MALDI-TOF MS has the power to distinguish molecular weight differences due to extent of hydroxylation (Δ 16 amu), nature of interflavan bonds (A-type or B-type; Δ 2 amu), and substitutions such as glucosylation (Δ 162 amu), it lacks the ability to assign specific stereochemistry to the molecule.

On the basis of the structures described by Gupta and Haslam (*13*) and later by Brandon et al. (*14*), an equation was formulated to predict heteropolyflavan-3-ols of a higher DP (**Table 1**). The equation is 290 +288*a* + 304*b* + cation, where 290 represents the molecular weight of the terminal catechin unit, *a* is the DP contributed by the repeating catechin/epicatechin unit (**Figure 1**), *b* is the DP contributed by the gallocatechin/epigallocatechin unit (**Figure 1**), and cation is the molecular weight of Na^+, K^+, Ag^+, or Cs^+ (23, 39, 107/109, or 133, respectively).

On the basis of the structures described by Gujer et al. (*15*), an equation was formulated to predict the glucosylated heteropolyflavans with eriodictyol or eriodictyol-5-*O*-β-glucoside as the terminal unit (**Table 2**). This equation is 288 + 272*a* +256*b* +162*c* + cation, were 288 represents the molecular weight of the terminal flavanone (eriodictyol) unit, *a* is the DP contributed by the flavan unit with hydroxylations at the 3′ and 4′ positions of the B-ring (proluteolinidin; **Figure 1**), *b* is the DP contributed by the flavan unit with one hydroxylation at the 4′ position of the B-ring (proapigeninidin; **Figure 1**), *c* is the number of

Table 1. Observed and Calculated Masses[a] of Heteropolyflavan-3-ols by MALDI-TOF MS

polymer length	no. of catechin flavan-3-ol units	no. of gallocatechin flavan-3-ol units	no. of A-type[b] bonds	no. of B-type bonds	calculated $[M + Cs]^+$	observed $[M + Cs]^+$
tetramer	4	0	0	3	1287	1287
	4	0	1	2	1285	1285
	4	0	2	1	1283	1283
	4	0	3	0	1281	1281
	3	1	0	3	1303	1303
	3	1	1	2	1301	1301
	3	1	2	1	1299	1299
	3	1	3	0	1297	1297
	2	2	0	3	1319	1319
	2	1	1	2	1317	1317
	2	1	2	1	1315	1315
	2	1	3	0	1313	1313
pentamer	5	0	0	4	1575	1575
	5	0	1	3	1573	1573
	5	0	2	2	1571	1571
	5	0	3	1	1569	1569
	5	0	4	0	1567	1567
	4	1	0	4	1591	1591
	4	1	1	3	1589	1589
	4	1	2	2	1587	1587
	4	1	3	1	1585	1585
	4	1	4	0	1583	1583
	3	2	0	4	1607	1607
	3	2	1	3	1605	1605
	3	2	2	2	1603	1603
	3	2	3	1	1601	1601
	3	2	4	0	1599	1599
hexamer	6	0	0	5	1863	1863
	6	0	1	4	1861	1861
	6	0	2	3	1859	1859
	6	0	3	2	1857	1857
	6	0	4	1	1855	1855
	6	0	5	0	1853	1853
	5	1	0	5	1879	1879
	5	1	1	4	1877	1877
	5	1	2	3	1875	1875
	5	1	3	2	1873	1873
	5	1	4	1	1871	1871
	4	2	0	5	1895	1895
	4	2	1	4	1893	1893
	4	2	2	3	1891	1891
	4	2	3	2	1889	1889
	4	2	4	1	1887	1887
heptamer	7	0	0	7	2151	2151
	6	1	0	7	2167	2167
	5	2	0	7	2183	2183
octamer	8	0	0	8	2439	2439
	7	1	0	8	2455	2455
	6	2	0	8	2471	2471
nonamer	9	0	0	9	2727	2727
	8	1	0	9	2743	2743
	7	2	0	9	2759	2759

[a] Mass calculations were based on the equation $290 + 288a + 304b + 133$, where 290 is the molecular weight of the terminal catechin unit, a is the degree of polymerization (DP) contributed by the catechin extending unit, b is the DP contributed by the gallocatechin extending unit, and 133 is the atomic weight of cesium. [b] Formation of each A-type interflavan ether linkage leads to the loss of two hydrogen atoms (2 amu).

glucose units, and cation is the molecular weight of Na^+, K^+, Ag^+, or Cs^+ (23, 39, 107/109, or 133, respectively).

Heteropolyflavan-3-ols with B-Type and A-Type Interflavan Bonds. The nomenclature for "polyflavan-3-ols" or "proanthocyanidins" is derived from the acid-catalyzed autooxidation reaction that produces anthocyanidins upon heating polyflavans in acidic alcohol solutions (*17*). The most common anthocyanidins produced are cyanidin and delphinidin from the corresponding polyflavan-3-ols procyanidin and prodelphinidin. This nomenclature, along with much of the earlier research on polyflavans, implies that these compounds are primarily homopolymers with uniform interflavan bonds and monomeric units.

Table 2. Observed and Calculated Masses[a] of Glucosylated Heteropolyflavans by MALDI-TOF MS

polymer length[b]	no. of proluteolinidin flavan units	no. of proapigeninidin flavan units	calculated $[M + Cs]^+$	observed $[M + Cs]^+$
trimer + 2 glucose	0	2	1257	1257
	1	1	1273	1273
	2	0	1289	1289
trimer + 3 glucose	0	2	1419	1419
	1	1	1435	1435
	2	0	1451	1451
tetramer + 3 glucose	0	3	1675	1676
	1	2	1691	1691
	2	1	1707	1707
	3	0	1723	1723
tetramer + 4 glucose	0	3	1837	1837
	1	2	1853	1853
	2	1	1869	1869
	3	0	1885	1885
pentamer + 4 glucose	0	4	2093	2093
	1	3	2109	2109
	2	2	2125	2125
	3	1	2141	2141
	4	0	2157	2157
pentamer + 5 glucose	0	4	2256	2255
	1	3	2272	2271
	2	2	2288	2287
	3	1	2304	2303
	4	0	2320	2319
hexamer + 5 glucose	0	5	2512	2512
	1	4	2528	2528
	2	3	2544	2544
	3	2	2560	2560
	4	1	2576	2576
	5	0	2592	2592
hexamer + 6 glucose	0	5	2674	2675
	1	4	2690	2690
	2	3	2706	2706
	3	2	2722	2722
	4	1	2738	2738
	5	0	2754	2754
heptamer + 6 glucose	0	6	2930	2931
	1	5	2946	2945
	2	4	2962	2962
	3	3	2978	2978
	4	2	2994	2995
	5	1	3010	3009
	6	0	3026	3025
heptamer + 7 glucose	0	6	3092	3033
	1	5	3108	3108
	2	4	3124	3124
	3	3	3140	3140
	4	2	3156	3156
	5	1	3172	3172
	6	0	3188	3188

[a] Mass calculations were based on the equation $288 + 272a + 256b + 162c + 133$, where 288 is the molecular weight of the terminal flavanone (eriodictyol) unit, a is the degree of polymerization (DP) contributed by the proluteolinidin extending unit, b is the DP contributed by the proapigeninidin extending unit, c is the number of glucose units, and 133 is the atomic weight of cesium. [b] Eriodictyol as terminal unit.

The MALDI-TOF spectrum of the Sephadex−methanol eluate showed a series polyflavan-3-ols extending from the tetramer (m/z 1287) to the nonamer (m/z 2727) in positive-ion reflectron mode (**Figure 2**). The Sephadex−80% aqueous acetone eluate contained masses corresponding to an oligomeric series of polyflavan-3-ol units extending from the pentamer (m/z 1575)

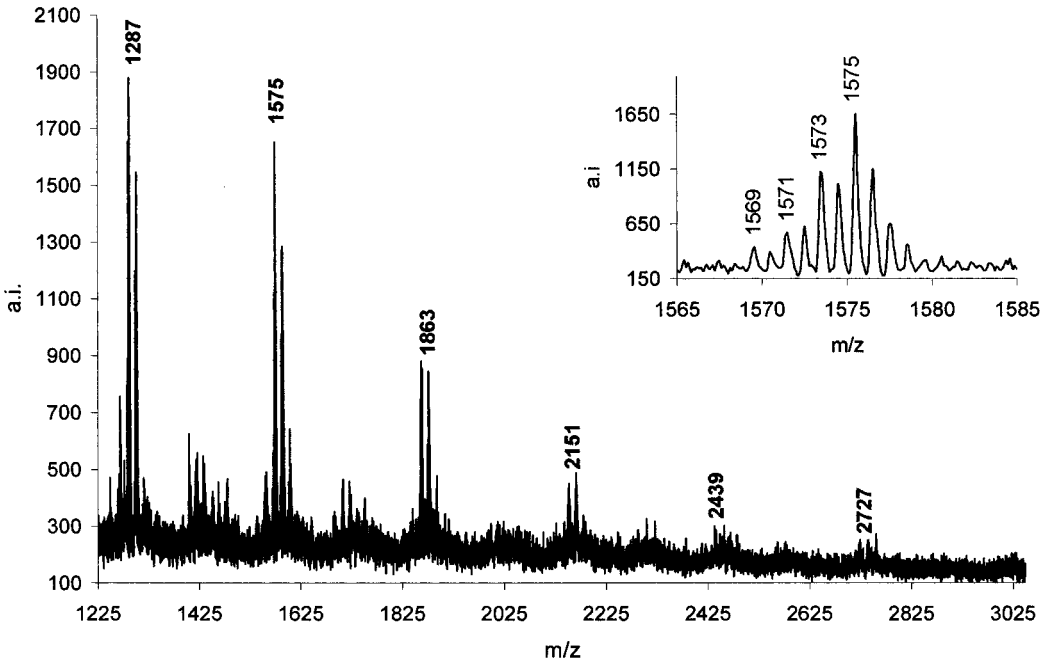

Figure 2. MALDI-TOF positive reflectron mode mass spectra of the Sephadex LH20-methanol eluate of Ruby Red sorghum [*Sorghum bicolor* (L.) Moench]. Masses represent the catechin/epicatechin homopolymer of the polyflavan-3-ol series $[M + Cs]^+$. The inset is an enlarged spectrum of the polyflavan-3-ol pentamer representing A-type and B-type interflavan bonds.

to the 13mer (*m*/*z* 3879) in positive-ion reflectron mode and up to the 20mer (*m*/*z* 5895) in positive-ion linear mode.

In addition to the predicted homopolyflavan-3-ol mass series seen in both the Sephadex−methanol and Sephadex−80% aqueous acetone eluates, each DP had a subset of masses Δ 16 amu and Δ 32 amu higher (**Figure 3**). These masses can be explained by heteropolymers of repeating flavan-3-ol units containing an additional hydroxyl group (Δ 16 amu) at the 5′ position of the B-ring as described by Brandon et al. (*14*). Each DP also had a subset of masses Δ 16 amu lighter than the predicted homopolymer (**Figure 3**); although this series of masses was not predicted by previously described monomeric units, it is speculated that mass assignments represent a further degree of heterogenity in which the compound is one hydroxyl substitiution (Δ 16 amu) lighter than the predicted mass (**Table 1**).

Positive reflectron mode mass spectra also shows a series of compounds that are Δ 2 amu multiples lower than those described in the predictive equation for heteropolyflavan-3-ols (**Figure 2**). These masses might represent a series of compounds in which the A-type interflavan ether linkage occurs (4β-8, 2β-*O*-7) between adjacent flavan-3-ol subunits because two hydrogen atoms (Δ 2 amu) are lost in the formation of this interflavan bond (**Table 1**).

A similar mass distribution has been reported to occur in cranberries (*7*), a fruit that contains A-type linkages (*3*). Takahata et al. (*11*) reported the presence of this mass distribution in brown and black soybeans and ascertained that these peaks did not originate from fragmentation or dehydration by applying MALDI-TOF MS to a sample of commercially available procyanidin C1 [epicatechin-(4B-8)-epicatechin-(4B-8)-epicatechin] that did not contain ions with masses Δ 2 amu lower than predicted.

Ion Adducts. There is a second explanation for the detection of Δ 16 amu differences in the mass spectrum that must be addressed. MALDI-TOF mass spectra of polyflavans tend to favor an association with naturally abundant sodium $[M + Na]^+$ and potassium $[M + K]^+$ ions over the formation of a protonated molecular ion $[M + H]^+$ (*7*, *11*). If both Na^+ and K^+ are present at the time of the desorption/ionization event, the signal of the polyflavan will be split and detected as both $[M + Na]^+$ and

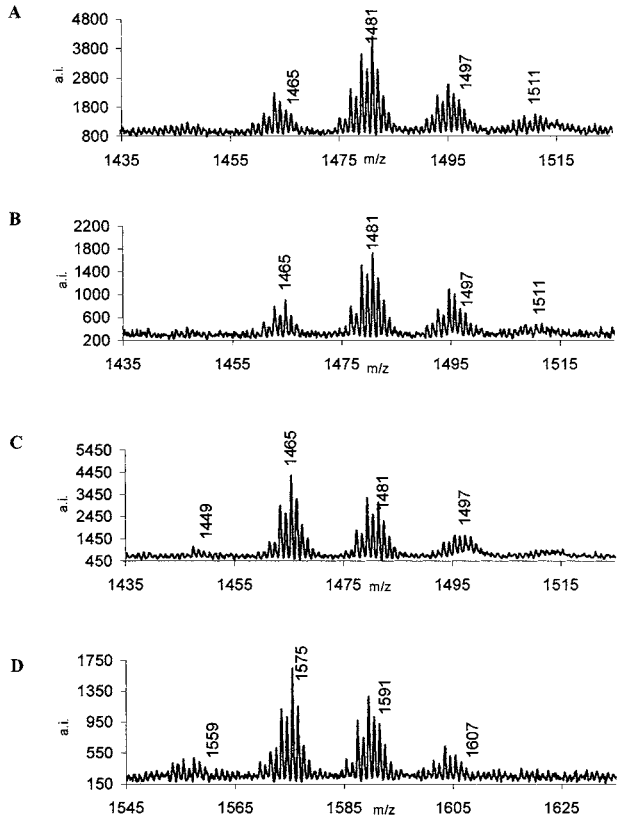

Figure 3. MALDI-TOF positive reflectron mode mass spectra of the heteropolyflavan-3-ol pentamers in the Sephadex LH20–methanol eluate of Ruby Red sorghum [*Sorghum bicolor* (L.) Moench]. (A) No deionization or addition of cation to the matrix/analyte prior to deposition on the target. (B) Deionization and addition of K^+. (C) Deionization and addition of Na^+. (D) Deionization and addition of Cs^+ provides evidence that the mass distribution (Δ 16 amu) is due to heterogeneity of the repeating flavan-3-ol units.

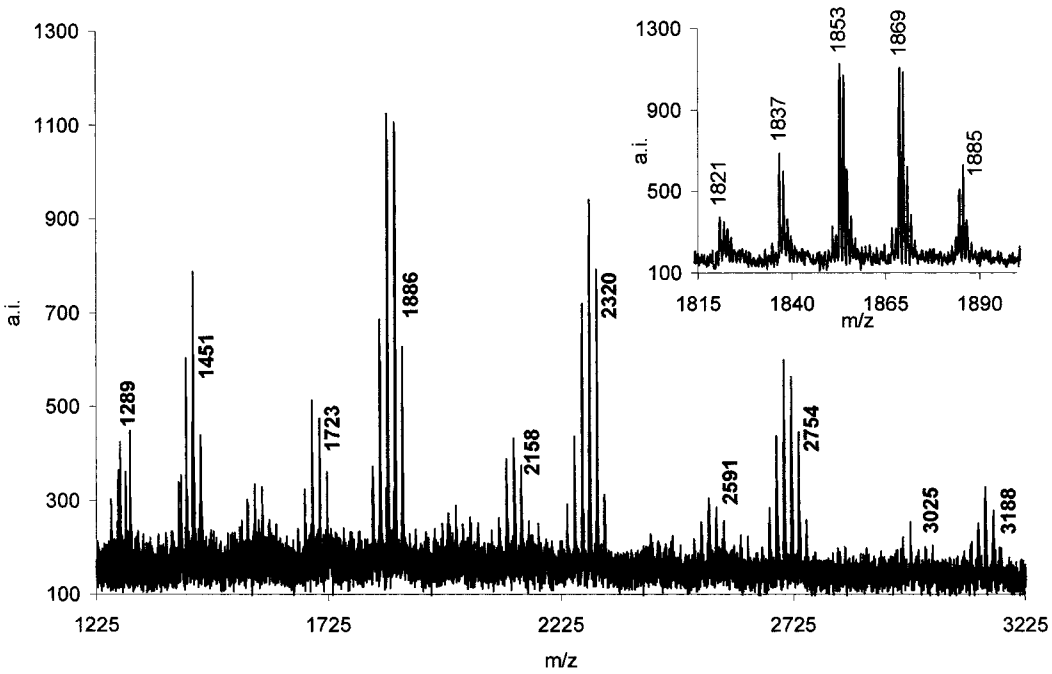

Figure 4. MALDI-TOF positive reflectron mode mass spectra of the Sephadex LH20-ethanol/methanol eluate of Ruby Red sorghum [*Sorghum bicolor* (L.) Moench]. Masses represent the polyflavan homopolymers $[M + Cs]^+$. The inset is an enlarged spectrum of the heteropolyflavan tetramers.

$[M + K]^+$ adduct ions. The atomic mass difference between the monoisotope of Na^+ (22.9900 amu) and the monoisotope of K^+ (39.0980amu) is Δ 15.9739 amu. The molecular weight difference of two polyflavans differing by one hydroxyl group substitution is equal to the atomic mass of oxygen (15.9949 amu).

To solve the problem of distinguishing between the formation of both $[M + Na]^+$ and $[M + K]^+$ adduct ions from one species, and the presence of two species differing in the number of hydroxyl groups, the Sephadex−methanol analyte was first mixed with matrix and applied directly to the target with no deionization or addition of cations. The predicted mass value for a homopolyflavan-3-ol with a DP of 5 and all B-type interflavan bonds is *m*/*z* 1481 ($[M + K]^+$) assuming that naturally abundant K^+ are the most abundant ions. The predicted value was observed as the most abundant mass (*m*/*z* 1481; $[M + K]^+$). However, masses 16 amu lighter and 16 and 32 amu heavier (*m*/*z* 1465, 1497, and 1511) than the predicted homopolymer were also observed (**Figure 3**). To determine whether these masses were due to heterogeneity in hydroxyl substitution (Δ 16 amu) or multiple cation adducts ($[M + Na]^+$ and $[M + K]^+$), the Sephadex−methanol analyte was deionized with cation-exchange resin, and K^+ was added. The mass spectra were found to give a mass distribution similar to that of the original spectra, with the predicted value *m*/*z* 1481 ($[M + K]^+$) remaining as the most abundant mass (**Figure 3**).

Ambiguity remained as to whether the mass at (*m*/*z* 1465) was due to a compound containing one fewer hydroxyl substitutions (Δ 16 amu) than the predicted homopolymer or the inability of the cation-exchange resin to remove all Na^+ (*m*/*z* 1465; $[M + Na]^+$). The Sephadex−methanol analyte was again deionized, and this time Na^+ was added. The predicted mass value for a homopolyflavan-3-ol with a DP of 5 and all B-type interflavan bonds is now *m*/*z* 1465 ($[M + Na]^+$), assuming that Na^+ is the only ion present. The entire mass distribution shifted Δ 16 amu lower than the original spectra (**Figure 3**). The predicted value was again observed as the most abundant mass (*m*/*z* 1465; $[M + Na]^+$). Again, masses 16 amu lighter and 16 and 32 amu heavier (*m*/*z* 1449, 1481, and 1497) than the predicted homopolymer were also observed (**Figure 3**). Ambiguity remained as to whether the mass at *m*/*z* 1481 was due to a compound containing one greater hydroxyl substitution (Δ 16 amu) than the predicted homopolymer or the inability of the cation-exchange resin to remove all K^+ (*m*/*z* 1481; $[M + K]^+$). To overcome this problem, a cation must be added to the analyte such that the mass distribution is shifted sufficiently from the range in which $[M + K]^+$ and $[M + Na]^+$ confound interpretation.

Onishi-Kameyama et al. (*12*) and Takahata et al. (*11*) employed silver trifluoroacetate to suppress cationization with Na^+ and K^+ $[M + \text{alkali metal}]^+$. However, the addition of Ag^+ created two new problems. Silver has two naturally occurring isotopes: ^{107}Ag (51.839% abundance) and ^{109}Ag (48.161% abundance). The addition of Ag^+ will effectively split the signal of the analyte, leading to the detection of $[M + Ag]^+$ ions differing by 2 amu. This is particularly confusing for analysis of polyflavans containing both B-type and A-type interflavan linkages (*7*, *11*). A second disadvantage of Ag^+ is the production of silver cluster ions in the presence of acidic matrixes such as *t*-IAA. Silver clusters formed preferentially, as opposed to silver-adducted oligomer ions (*18*).

To eliminate the problem of cationization with Na^+ and K^+, we first deionized the polyflavan solutions by adding a cation-exchange resin and then added cesium trifluoroacetate to the analyte/matrix solution prior to deposition on the target. Cesium has the attribute of having a single isotope (^{133}Cs, 100% abundance). This approach resulted in the detection of exclusively $[M + Cs]^+$ molecular ions (**Figure 3**), eliminating the possibility that the Δ 16 amu signal was due to the formation of both $[M + Na]^+$ and $[M + K]^+$ adduct ions from one species. The mass distribution provides evidence for a class of heteropolyflavan-3-ols that have one fewer hydroxyl substitution (Δ 16 amu) than the predicted equations developed on the basis of the structures previously described by Gupta and Haslam (*13*) and later by Brandon et al. (*14*) and also provides evidence for the presence of gallocatechin/epigallocatechin hydroxylation patterns (**Table 1**).

Glucosylated Heteropolyflavans with Eriodictyol/Eriodictyol-5-*O*-*β*-glucoside Terminal Unit. In the case of sor-

ghum, Gujer et al. (*15*) elucidated the dimeric and trimeric structures of a unique class of heteropolymeric flavonoids. This class of flavonoids consisted of repeating monomeric flavan units glucosylated at carbon-5 and contained a flavanone, eriodictyol or eriodictyol-5-*O*-*β*-glucoside, as the terminal unit (**Figure 1**). We also detected heterogeneity in the pattern of hydroxylation in the B-ring by heating the Sephadex LH20−ethanol/methanol eluate with acid (*17*), separating reaction products on a polyvinyl polypyrrolidone column and subjecting the anthocyanins to MALDI-TOF MS. The anthocyanins produced were luteolinidin (*m*/*z* 271) and apigeninidin (*m*/*z* 255).

The positive-ion reflectron mode MALDI-TOF spectrum of the Sephadex−ethanol/methanol eluate contained masses corresponding to two distinct oligomeric series of glucosylated heteropolyflavan units (**Table 2**). The first series represents glucosylated heteropolyflavans containing the flavanone eriodictyol as the terminal unit. The mass distribution extends from the trimer (*m*/*z* 1289) to the heptamer (*m*/*z* 3025) (**Figure 4**). The second series, separated by a glucose substitution (Δ 162 amu), represents glucosylated heteropolyflavans containing the flavanone eriodictyol 5-*O*-*β*-glucoside as the terminal unit and extends from the trimer (*m*/*z* 1451) to the heptamer (*m*/*z* 3188) (**Figure 4**).

Each DP within the glucosylated heteropolyflavan mass series had a subset of masses (Δ 16 amu) lower than the proluteolinidin oligomer in which all glucosylated flavan units are hydroxylated at both the 3′ and 4′ positions of the B-ring (**Figure 1**). In the case of the tetramer, four iterations are predicted and observed: *m*/*z* 1837 (3 proluteolinidin + 0 proapigeninidin), *m*/*z* 1853 (2 proluteolinidin + 1 proapigeninidin), *m*/*z* 1869 (1 proluteolinidin + proapigeninidin), and *m*/*z* 1885 (0 proluteolinidin + 3 proapigeninidin) each with eriodictyol 5-*O*-*β*-glucoside as the terminal unit (**Figure 1b** and **Table 2**). The mass differences are explained by the hydroxyl substitutions (Δ 16 amu) at either the 3′ or 3′ and 4′ positions of the B-ring of the repeating flavan unit. In addition, a mass 16 amu lower than the proapigeninidin homopolyflavan was also detected at each DP. Although no predictive equation was formulated to account for these masses, it is speculated that the masses represent a compound with one fewer hydroxyl (Δ 16 amu) substitution.

The combination of liquid chromatographic separation and MALDI-TOF MS to characterize sorghum polyflavans indicates that the structural heterogeneity is much greater than previously described. Structural heterogeneity occurs in the nature of repeating monomeric units (flavan, flavan-3-ol and flavanone), pattern of hydroxylation, type of interflavan bonds (A-type and B-type), and substitutions with moieties such as glucose. In light of this structural diversity a more descriptive nomenclature must be employed to describe heteropolyflavans. The ability to characterize the structural diversity of polyflavans in foods is required to better determine their effects on nutrition and health.

ABBREVIATIONS

MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *t*-IAA, *trans*-3-indoleacrylic acid; DP, degree of polymerization; amu, atomic mass unit.

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LITERATURE CITED

(1) Price, M. L.; Hagerman, A. E.; Butler, L. G. Tannin in sorghum grain: Effect of cooking on chemical assays and antinutritional properties in rats. *Nutr. Rep. Int.* **1980**, *21*, 761−767.

(2) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds—Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 094−1117.

(3) Foo, L. Y.; Lu, Y.; Howell, A. B.; Verosa, N. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic p-fimbriated *Eschericia coli*. *J. Nat. Prod.* **2000**, *63*, 1225−1228.

(4) Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **2000**, *130*, 2086S−2092S.

(5) Hanton, S. D. Mass spectrometry of polymers and polymer surfaces. *Chem. Rev.* **2001**, *101*, 527−569.

(6) Montaudo, G.; Montaudo, M. S.; Samperi, F. Matrix-assisted laser desorption ionization/mass spectrometry of polymers (MALDI-MS). In *Mass Spectrometry of Polymers*; Montaudo, G., Lattimer, R. P., Eds.; CRC Press: Boca Raton, FL, 2002; pp 419−521.

(7) Porter, M. L.; Krueger, C. G.; Wiebe, D. A.; Cunningham, D. G.; Reed, J. D. Cranberry proanthocyanidins associate with low-density lipoprotein and inhibit in vitro Cu^{2+}-induced oxidation. *J. Sci. Food Agric.* **2001**, *81*, 1306−1313.

(8) Krueger, C. G.; Dopke, N.; Treichel, P. M.; Folts, J.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.* **2000**, *48*, 1663−1667.

(9) Yang, Y.; Chien, M. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem.* **2000**, *48*, 3990−3996.

(10) Hedqvist, H.; Mueller-Harvey, I.; Reed, J. D.; Krueger, C. G.; Murphy, M. Characterisation of tannins and in vitro protein digestibility of several *Lotus corniculatus* varieties. *Anim. Feed Sci. Technol.*. **2000**, *87*, 41−56.

(11) Takahata, Y.; Ohnishi-Kameyama, M.; Furuta, S.; Takahashi, M.; Suda, I. Highly polymerized procyanidins in brown soybean seed coat with a high radical-scavenging activity. *J. Agric. Food Chem.* **2001**, *49*, 5843−5847.

(12) Ohnishi-Kameyama, M.; Yanagida, A.; Kanda, T.; Nagata, T. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 31−36.

(13) Gupta, R. K.; Haslam, E. *J. Chem Soc., Perkin Trans*. **1978**, *1*, 892.

(14) Brandon, M. J.; Foo, L. Y.; Porter, L. J.; Meredith, P. Proanthocyanidins of barley and sorghum composition as a function of maturity of barley ears. *Phytochemistry* **1982**, *21*, 2953−2957.

(15) Gujer, R.; Magnalato, D.; Self, R. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry* **1986**, *25*, 1431−1436.

(16) Price, M. L.; Van Scoyoc, S.; Butler, L. G. A critical evaluation of the vannilin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem*. **1978**, *26*, 1214−1218.

(17) Bate-Smith, E. C.; Rasper, V. Tannins of grain sorghum: luteoforol (leucoluteolinidin), 3′,4,4′,5,7-pentahydroxyflavan. *J. Food Sci.* **1969**, *34*, 203−209.

(18) Macha, S. F.; Limbach, P. A.; Hanton, S. D.; Owens, K. G. Silver cluster interferences in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry of nonpolar polymers. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 732−743.

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